Halopiger aswanensis sp. nov., a polymer-producing and extremely halophilic archaeon isolated from hypersaline soil

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Strain 56^T was isolated from a hypersaline soil in Aswan (Egypt). Cells were pleomorphic rods. The organism was neutrophilic, motile and required at least 1.7 M (10 % w/v) NaCl, but not MgCl₂, for growth; optimal growth occurred at ≥3.8 M (≥22.5 %) NaCl. The strain was thermotolerant with an optimum temperature for growth of 40 °C, although growth was possible up to 55 °C. The G+C content of the DNA of the novel strain was 67.1 mol%. 16S rRNA gene sequence analysis revealed that strain 56^T was a member of the phyletic group defined by the family *Halobacteriaceae*, showing the highest similarity to *Halopiger xanaduensis* SH-6^T (99 %) and the next highest similarity of 94 % to other members of the family *Halobacteriaceae*. DNA–DNA hybridization revealed 27 % relatedness between strain 56^T and *Hpg. xanaduensis* SH-6^T. Polar lipid analysis revealed the presence of the bis-sulfated glycolipid S₂-DGD-1 as the sole glycolipid and the absence of the glycerol diether analogue phosphatidylglycerosulfate. Both C_{20.20} and C_{20.25} core lipids were present. Strain 56^T accumulated large amounts of polyhydroxybutyrate and also secreted an exopolymer. Physiological and biochemical differences suggested that *Hpg. xanaduensis* and strain 56^T were sufficiently different to be separated into two distinct species. It is suggested that strain 56^T represents a novel species of the genus *Halopiger*, for which the name *Halopiger aswanensis* sp. nov. is proposed. The type strain is strain 56^T (=DSM 13151^T=JCM 11628^T).

The genus *Halopiger* was proposed by Gutiérrez *et al.* (2007) and currently contains one species, *Halopiger xanaduensis*. The recently described type species *Hpg. xanaduensis* is Gram-negative and its cells are pleomorphic, but mostly form long rods. In an ongoing study, microorganisms from a soil sample collected from the surface of hypersaline soils close to Aswan, Egypt, were screened with

respect to endo- and exopolymer formation. This study has already resulted in the isolation of the type species *Halobiforma haloterrestis* (Hezayen *et al.*, 2002a), which accumulates polyhydroxyalkanoate, and *Natrialba aegyptiaca* (Hezayen *et al.*, 2001) which produces poly(γ-glutamic acid) (Hezayen *et al.*, 2000). This paper reports the isolation of a pink-pigmented, extremely halophilic strain, strain 56^T, from the same hypersaline soil sample. Strain 56^T produced polyhydroxyalkanoate and exopolymers. The mechanism for polyhydroxyalkanoate production and the biosynthesis enzymes involved have already been investigated in more detail (Hezayen *et al.*, 2000, 2002b). 16S rRNA gene sequence analysis suggested that strain 56^T was closely related to *Hpg. xanaduensis*. The aim of this study was to clarify the taxonomic status of this

Abbreviations: Me-PGP, methylated phosphatidylglycerolphosphate; PG, phosphatidylglycerol; PGS, phosphatidylglycerosulfate; S₂-DGD, bis-sulfated diglycosyl diether.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 56^T is AF333759.

A TLC of the polar lipids of strain 56^T and reference organisms is available as a supplementary figure with the online version of this paper.

isolate and it is proposed that strain 56^T represents a novel species of the genus *Halopiger*.

Strain 56^T was isolated and grown on Sehgal–Gibbons (S–G) medium (Sehgal & Gibbons, 1960). The basal medium contained the following (l⁻¹): 225 g NaCl, 2 g KCl, 5 g MgSO₄·7H₂O and 1 g yeast extract, and was used for physiological and biochemical characterization. The salt mineral medium, which contained (l⁻¹): 250 g NaCl, 2 g KCl, 5 g MgSO₄·7H₂O, 7.5 g proteose peptone and 5 g yeast extract, was used to test the utilization of nitrogenous or non-nitrogenous substrates as sole carbon and energy source. For non-nitrogenous substrates, the medium was supplemented at a concentration of 0.2 % (w/v) according to the methods of Hezayen *et al.* (2000, 2001, 2002a). If required, all media were solidified by adding 2 % (w/v) agar before autoclaving. The pH was adjusted with NaOH.

Phenotypic tests were performed according to the proposed minimal standards for the description of new taxa in the order of *Halobacteriales* (Oren *et al.*, 1997). Gram staining was performed by using acetic acid fixed samples as described by Dussault (1955). Wet mounts were prepared for motility determination and examination of cell morphology at various growth stages from both broth and solid media at different NaCl concentrations. Observations were made with an Ortholux II optical microscope (Leitz). The growth responses of the novel strain with varying NaCl concentrations, pH and incubation temperatures were determined in liquid S–G medium and were monitored spectrophotometrically. Catalase and oxidase activities, other enzymic activities, acid production from carbohydrates and all other physiological and biochemical characteristics were performed according to Gerhardt *et al.* (1994) and modified as described previously (Hezayen *et al.*, 2001). Utilization of several different substrates as carbon and energy source was investigated as previously described (Hezayen *et al.*, 2002a). Phenotypic characteristics of strain 56^T are summarized in Table 1 and in the species description.

Cells of strain 56^T were Gram-negative and motile and formed slightly pink colonies on S–G medium. The intensity of pigmentation increased when cells grown on agar plates were then exposed to daylight at room temperature for at least one week. The pigmentation was due to carotenoid production. Carotenoids were extracted from cells grown in liquid and solid S–G medium at 1.7 M (10 % w/v) and 5.2 M (25 % w/v) NaCl, respectively, and were analysed according to the methods of Gochner *et al.* (1972). Extracted carotenoids showed absorption maxima at wavelengths of 370, 390, 496 and 528 nm with a shoulder at 466–476 nm. These maxima corresponded to those of bacterioruberin (Gochner *et al.*, 1972). Colonies were small and circular, up to 1 mm in diameter, opaque, convex and sticky due to slime production. Cells of strain 56^T growing exponentially in liquid medium under optimal conditions were rod-shaped and pleomorphic and measured 1.25–6.5 × 0.6–0.9 µm, while cells from agar plates were shorter and measured 0.9–3.75 in length (Fig.

Table 1. Characteristics that distinguish strain 56^T from the type strain of *Hpg. xanaduensis*

Strains: 1, 56^T; 2, *Hpg. xanaduensis* SH-6^T. –, Negative; +, positive; w, weakly positive; R, resistant; S, susceptible.

Characteristic	1	2
Cell size (µm)	3–13 × 0.5–1	1.25–6.5 × 0.6–0.9
Pigmentation	Pink	Red
NaCl optimum (M)	3.8–4.3	4.3
NaCl range (M)	1.7–5.2	2.5–5.0
pH optimum	7.5	8.0
Temperature optimum (°C)	40	37
Max. growth temperature (°C)	55	45
Acid production from:		
Fructose	+	–
Sucrose	+	–
Maltose	+	–
Nitrite from nitrate	+	–
Hydrolysis of:		
Starch	+	–
Gelatin	–	+
Tween 80	w	+
Indole production	+	–
Urease	–	+
H ₂ S production	+	–
Rifampicin	S	R
DNA G + C content (mol%)	67.1	63.1

1). Other phenotypic characteristics of strain 56^T are summarized in Table 1 and in the species description.

Detection of diether lipids, extraction of respiratory lipoquinones and polar lipids and the analysis of polar lipids were performed according to Tindall (1990a, b) as well as previously described methods (Hezayen *et al.*, 2001). Lipid analysis showed that strain 56^T was a member of the family *Halobacteriaceae*. The ether lipids comprised only diether lipids, tetraether lipids being absent. The diether lipids present were the diphytanyl derivatives (C_{20·20} and C_{20·25}). Menaquinones were the only respiratory lipoquinones present and comprised MK-8 (67.5 %) and MK-8(VIII-H₂) (32.5 %). The polar lipid analysis revealed the presence of phosphatidylglycerol (PG) and methylated phosphatidylglycerolphosphate (Me-PGP), whereas no phosphatidylglycerosulfate (PGS) was detected. Furthermore, a bis-sulfated diglycosyl diether (S₂-DGD) was produced, which is absent in the majority of neutrophilic and from all alkaliphilic members of the order *Halobacteriales* (see Supplementary Fig. S1 in IJSEM Online). The lipid analysis and polar lipid data indicated that strain 56^T was a member of the family *Halobacteriaceae* with a specific evolutionary relationship with the phyletic group defined by members of the genera *Halopiger*, *Halobiforma*, *Natronococcus*, *Natrialba*, *Natrinema*, *Haloterrigena* and *Natronorubrum*.

The DNA G + C content was determined using hydrolysed DNA using the methods of Cashion *et al.* (1977) and Mesbah *et al.* (1989) and as previously described by Hezayen *et al.*

Fig. 1. Phase-contrast micrographs of cells of strain 56^T grown on agar plates (a) and liquid S–G medium (b) at 25 % (w/v) NaCl. Bars, 5 µm.

(2001). Chromosomal DNA was extracted from cells lysed in distilled water by phenol/chloroform extraction followed by ethanol precipitation modified according to the methods of Mak & Ho (1992). The G + C content of the DNA of strain 56^T was 67.1 mol% (mean of three independent determinations), which differed significantly from the value of ~63.1 mol% for the most related member of the family Halobacteriaceae, *Hpg. xanaduensis*.

The 16S rRNA gene was amplified by PCR (forward primer 5'-CCGGAGGTCATTGCTAGTGGAGTC-3' (corresponding to *Halobacterium cutirubrum* NCIMB 763, positions 16–40) and the reverse primer 5'-AGGAGGTGATCCAGCCGCAGATTCC-3' (corresponding to *Hbt. cutirubrum* NCIMB 763, positions 1472–1448) using purified chromosomal DNA as the template. The resulting PCR product was cloned into vector pGEM-T Easy (Promega), and the recombinant plasmid was propagated in *Escherichia coli* XL1-Blue (Stratagene). The near full-length DNA sequence (1456 bp) of the 16S rRNA gene was determined as described previously (Hezayen *et al.*, 2002a). Various methods of data analysis of the aligned sequences were used as suggested by Ludwig *et al.* (1998). The sequence was compared with closely related 16S rRNA gene sequences of reference organisms from the FASTA network service (<http://www.ebi.ac.uk/fasta33>). Multiple alignment was performed with CLUSTAL W, version 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed using the neighbour-joining method (Fig. 2). The following sequences were used: strain 56^T (GenBank accession no. AF333759), *Hpg. xanaduensis* SH-6^T (AM268114), *Natronorubrum tibetense* GA33^T (AB005656), *Natrialba asiatica* 172P1^T (D14123), *Natronococcus occultus* NCIMB 2192^T (Z28378), *Haloterrigena limicola* AX-7^T (DQ367241), *Natrinema versiforme* XF10^T (AB023426) and *Halobiforma nitratireducens* C231 (NR_028153). Analysis of the 16S rRNA gene sequence of strain 56^T indicated that it grouped with *Hpg. xanaduensis* (99 %) but not with any other recognized taxa within the family Halobacteriaceae. The sequence of strain 56^T exhibited the highest similarity of 94 % to members of the genera *Halobiforma*, *Natronococcus*, *Haloterrigena*, *Natrinema* and *Natrialba*.

DNA–DNA hybridization was carried out according to the competition procedure of Johnson (1994) and described in detail by Arahall *et al.* (2001). DNA was labelled by the multiprime system using a commercial kit with deoxy(1',2',5'-³H) cytidine 5'-triphosphate (Promega Corporation). Labelled DNA was denatured before hybrid-

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence comparisons showing the position of strain 56^T and some other related haloarchaeal species. GenBank accession numbers are indicated in parentheses. Bar, 0.01 substitutions per nucleotide position.

ization by incubation at 100 °C for 5 min and then placed on ice. Hybridization experiments were carried out under optimal conditions and at a temperature of 60 °C, which was within the suggested limits for the filter method (De Ley & Tijtgat, 1970). The DNA–DNA hybridization value of strain 56^T with *Hpg. xanaduensis* SH-6^T was 27%. In combination with the levels of 16S rRNA gene sequence similarity, this low DNA–DNA relatedness showed that strain 56^T was not genotypically related to this recognized species of the genus *Halopiger* (Stackebrandt & Goebel, 1994).

Strain 56^T has already been investigated as an endopolymer producer and it was found to accumulate polyhydroxybutyrate intracellularly as a reserve polymer which contributed up to 53% (w/v) of the cellular dry weight (Hezayen *et al.*, 2000, 2002b). Several members of the family *Halobacteriaceae* have been described as producing polyhydroxybutyrate as a reserve polymer (Fernandez-Castillo *et al.*, 1986; Lillo & Rodriguez-Valera 1990; Hezayen *et al.*, 2002a). In addition, strain 56^T produced an exopolymer which was composed of amino acids (65%, w/w), carbohydrates (15%, w/w) and unidentified compounds (20%, w/w). Exopolymer formation was observed by increased viscosity of the cultivation fluid when either starch, glucose or maltose were used as the sole carbon and energy source in the presence of NH₄Cl as the sole nitrogen source. Among the members of the family *Halobacteriaceae*, *Natrialba aegyptiaca*, a *Haloarcula* sp. and some members of the genus *Haloferax*, including *Haloferax mediterranei*, *Haloferax gibbonsii* and *Haloferax denitrificans*, have been described as exopolymer producers (Antón *et al.*, 1988; Paramonov *et al.*, 1998; Parolis *et al.*, 1999; Nicolaus *et al.*, 1999; Hezayen *et al.*, 2000, 2001).

The susceptibility of strain 56^T to antibiotics was assessed on surface-seeded S–G agar plates. The following compounds were tested (µg disc⁻¹ unless stated otherwise): penicillin G (5), chloramphenicol (10), tetracycline (30), streptomycin (25), nitrofurantoin (300), bacitracin (10 U), novobiocin (5), fucidic acid (10), anisomycin (20), trimethoprim (2.5), rifampicin (30), nystatin (100 U), nalidixic acid (30) and erythromycin (15). The antibiotic susceptibility data are summarized in the species description.

Strain 56^T is phylogenetically closest to *Hpg. xanaduensis*. However, it differed from this species in that *Hpg. xanaduensis* produces no acids from fructose, sucrose or maltose and is not able to hydrolyse starch, but can hydrolyse gelatin and Tween 80 (Table 1). In addition, *Hpg. xanaduensis* requires 2.5 M NaCl for growth, only grows up to a temperature of 45 °C, is resistant to rifampicin and has a lower DNA G+C content (63.1 mol%). Strain 56^T and *Hpg. xanaduensis* also showed differences in other important phenotypic traits such as nitrate reduction, indole production, urease activity and H₂S production (Table 1). Based on the above-mentioned significant differences between strain 56^T and *Hpg. xanaduensis*, it is concluded that strain 56^T represents a

novel species within the genus *Halopiger* for which the name *Halopiger aswanensis* sp. nov. is proposed.

Description of *Halopiger aswanensis* sp. nov.

Halopiger aswanensis (as.wan.en'sis. N.L. masc. adj. *aswanensis* isolated from a hypersaline soil in Aswan, Egypt).

Cells are motile, Gram-negative, pleomorphic rods (short and long) measuring 1.25–6.50 × 0.6–0.9 µm and 0.9–3.75 × 0.6–0.9 µm from liquid and solid cultivation media under optimum conditions, respectively. Colonies may be pink-pigmented and small. Cells require at least 1.7 M (10% w/v) NaCl for growth. Optimum growth occurs at 3.8–4.3 M (22.5–25%) NaCl with an optimum pH of 7.5 and an optimum temperature of 40 °C. Cells are able to grow over a pH range of 6–9.2. The maximum temperature for growth is 55 °C. Oxidase- and catalase-positive. Cells lyse in distilled water. Strictly aerobic. Anaerobic growth does not occur even in the presence of KNO₃ or arginine. Mg²⁺ is not required for growth. Indole was produced from tryptone, sulfide was produced from cysteine, and starch, Tween 20, Tween 40 and Tween 80 are hydrolysed. Casein, gelatin or lipids from egg yolk are not hydrolysed. Nitrate is reduced to nitrite. Acid is produced from xylose, arabinose, glucose, fructose, sucrose, maltose and starch, but not from lactose. Susceptible to fusidic acid, bacitracin, nitrofurantoin, novobiocin, anisomycin, trimethoprim and rifampicin. Resistant to nystatin, tetracycline, nalidixic acid, erythromycin, chloramphenicol, streptomycin and penicillin. Organic nitrogenous compounds, e.g. proteose peptone, tryptone, Casamino acids and non-nitrogenous compounds such as some carbohydrates and acetate, gluconate and pyruvate can be used as the carbon and energy source. Menaquinones are the only respiratory lipoquinones present and comprise MK-8 (67.5%) and MK-8(VIII-H₂) (32.5%). Polar lipid analysis shows the presence of the bis-sulfated glycolipid S₂-DGD-1 as the sole glycolipid and the glycerol diether analogue of phosphatidylglycerol sulfate is absent. Growth occurs with starch or other compounds as the sole carbon and energy source in the presence of NH₄Cl as the sole nitrogen source.

The type strain, strain 56^T (=DSM 13151^T=JCM 11628^T), was isolated from the surface of hypersaline salt soils close to Aswan, Egypt. The DNA G+C content of type strain is 67.1 mol%.

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